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Review

Regulation of pannexin channels by post-translational modifications

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ABSTRACT

The large-pore channels formed by the pannexin family of proteins have been implicated in many physiological and pathophysiological functions, mainly through their ATP release function. However, a tight regulation of channel opening is necessary to modulate their function in vivo. Post-translational modifications have been postulated as some of the regulating mechanisms for Panx1, while Panx2 and Panx3 have not been as well characterized. Positive regulators include caspase cleavage to open Panx1 channels in apoptotic cells, and activation by Src family kinases via ionotropic receptors in neurons and macrophages. S-nitrosylation of cysteines has been shown to both inhibit and activate the Panx1 channel in different cell types. All three pannexins are N-glycosylated but to different levels of modification. Their diverse glycosylation appears to regulate cellular localization, intermixing, and may restrict their ability to function as inter-cellular channels. It is clear that our understanding of pannexin post-translational modification and their role in channel function regulation is still in its infancy even a decade after their discovery.

1. Introduction

It's been more than a decade since the three pannexins (Panx1, Panx2 and Panx3) were discovered based on their limited homology to the invertebrate gap junction proteins (innexins) [1]. It seems clear from the 300 papers on a 'pannexin' search in PubMed that the main function of this branch of the gap-junction super family is the formation of large pore, non-selective channels [2]. All Panx isoforms are tetra-spanning membrane proteins (Fig. 1). It is thought that functional Panx1 and Panx3 channels are hexameric, similar to the connexins, whereas Panx2 channels are likely octomeric [3]. All three pannexins appear to be able to form channels capable of dye uptake [4] and more importantly, at least for Panx1 and Panx3, they are conduits of physiologically important molecules such as ATP [5,6], intracellular Ca^{++} [7,8], and even glucose uptake (Panx1) [9]. Panx1 channels are proposed to have large-pores (up to ~500 pS) that allow molecules of ~1.5 kDa in size, and therefore, need to be tightly regulated so as not to compromise the integrity of the cell [5]. Unlike connexin hemichannels, pannexins are not gated by extracellular calcium and can therefore be open under physiological conditions [10]. Panx1 channels are known to be opened at resting membrane potentials through mechanisms involving their mechanosensitivity [5,11] and by increasing extracellular potassium concentrations [5,12,13]. Therefore, regulation of channel opening is critical to keep these large-pore channels closed when not needed, and promptly activated when triggered.

Panx1 has been more extensively characterized, and its function in ATP release [5] through an interaction with purinergic receptors [14,15] has been proposed to have a role in the propagation of calcium waves. ATP and UTP released by Panx1 channels can also have an important immune function as "find-me" signals for the clearance of apoptotic cells [16]. Panx1-mediated ATP release has been suggested to regulate vascular tone [17] and mucociliary lung clearance [18]. On the other hand, Panx1 channel opening can be deleterious, contributing to cell death under ischemic conditions, seizures [13,19], and facilitating HIV-1 viral infection [20]. Recently, a functional complex of Panx1-P2X7 receptors was shown to regulate cell death of enteric neurons in inflammatory bowel diseases, resulting in abnormal gut motility [21]. Panx2 is involved in differentiation of neurons [22], and its channel function, together with Panx1 can contribute to ischemic brain damage as evident in double (Panx1 and Panx2) constitutive knockout mice [23]. Like Panx1, Panx3 has also been shown to be a conduit for ATP release [7] and is important in the differentiation of chondrocytes [6] and osteoblasts [7].

The pannexin isoforms have broad tissue distribution, with the individual members having both distinct and overlapping expression patterns. Panx1 demonstrates the broadest distribution of

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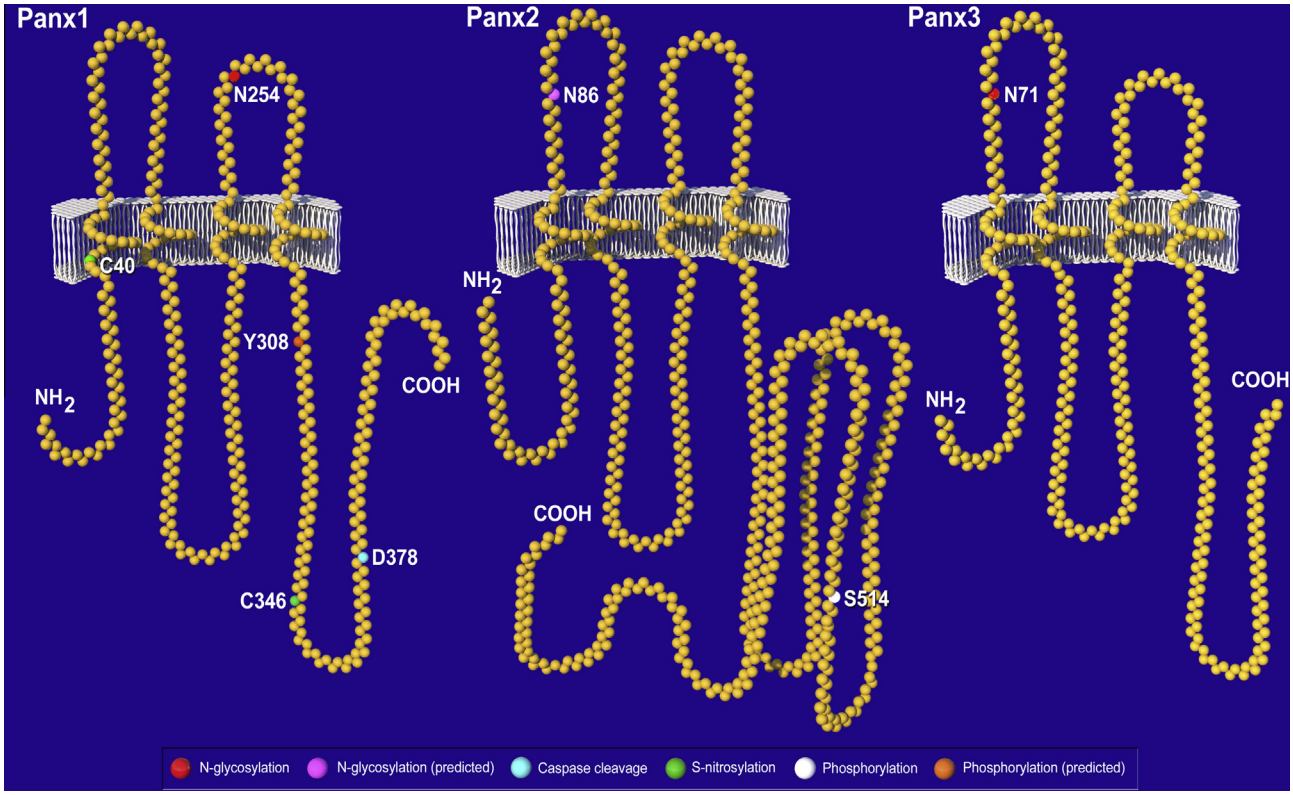

Fig. 1. Schematic representation of the three pannexin proteins. The pannexin family of channel-forming proteins has three members, Panx1 (A, 426 a.a.), Panx2 (B, 677 a.a.) and Panx3 (C, 392 a.a.). All pannexins are tetra-spanning membrane proteins with two extracellular loops and intracellular amino and carboxyl termini. Colored residues indicate sites of posttranslational modification, including N-glycosylation, caspase cleavage, S-nitrosylation and phosphorylation.

the pannexin family. Transcript expression of Panx1 has been reported for most tissues tested, with examples that include brain, testes, skeletal muscle, immune cells and skin [24]. Panx1 has been found in most glutamatergic principle cells in the brain, and is enriched in areas such as the hippocampus, amygdala, cortex, cerebellum and retina [24–28]. By way of comparison, Panx2 is thought to have restricted expression in the adult brain that overlaps with that of Panx1 [29–31]. Panx2 is also reportedly present in neural precursor (stem) cells [22]. Finally, Panx3 has its highest expression outside of the nervous system, with transcript and protein expression being reported in chondrocytes and osteoblasts [6,7,32], skin [11], mammary gland [32] and the male reproductive tract [33].

All three pannexins are predominantly localized to the cell surface, however, they have been reported in the cytoplasm and intracellular organelles of different cell types. This targeted localization is regulated at least in part by post-translational modifications that may determine not only the subcellular location but also the function of the pannexin channels at a particular site [34]. In this review, we will present the most prominent topics related to pannexin post-translational modifications that were discussed at a roundtable discussion during the 2013 International Gap Junction Conference in Charleston, SC, USA.

2. N-glycosylation

Addition of oligosaccharides to proteins at asparagine residues is called N-glycosylation, which is a critical posttranslational modification occurring in the ER and Golgi that regulates protein folding, quality control and trafficking [35]. All three pannexins are glycoproteins containing identified N-glycosylation consensus sites (Fig. 1). However, they present a diverse profile of N-linked glycosylation that goes from the characteristic endoplasmic reticulum high mannose glycosylation (Gly1, sensitive to endoglycanase H), to the more complex editing that happens in the Golgi (Gly2, sensitive to N-glycosidase F) [4,11,36]. Panx2, which has been reported to display a predominantly intracellular localization, appears to be present in Panx2 ectopically expressing HEK 293T cells as a Gly0 (unglycosylated) and Gly1 (high mannose glycosylated), which is consistent with the intra-cellular localization profile described [3,4,22,37]. However, it should be noted that at least in this overexpression setting and using cell surface biotinylation, a subpopulation of Panx2 was detected at the plasma membrane that was capable of dye uptake [4]. The amount of Panx2 protein at the surface was doubled when co-expressed with Panx1, indicating a potential interaction between the two pannexins [4]. Based on co-immunoprecipitation assays, the Gly0 and Gly1 forms of Panx1 and Panx2 are found in a complex when co-expressed, while the Gly2 form (complex glycosylation) of Panx1 does not seem to facilitate an interaction with Panx2, and it is predominantly observed at the cell surface. The same is true for co-IPs of Panx1 and Panx3, where the Gly0 and Gly1 forms of the proteins are able to interact but this does not happen between the complex Gly2 forms of the proteins [4]. This indicates that N-glycosylation not only regulates pannexin localization in the different compartments of the cell, but also the way they interact with other pannexin family members.

We and others have noticed that pannexin localization profiles are quite diverse in different tissues and cell types. Even the ectopic expression in different reference cell lines can look quite different with more intracellular localization in some versus a prominent cell surface decoration on others [38]. It has been hypothesized that the level of glycosylation can have an impact on the cellular location and potentially on the function of the

channel. Panx1 and Panx3 have been shown to localize and function in the endoplasmic reticulum as a calcium leak channel [7,8], which would be consistent with the intracellular localization that is observed in vivo, for instance in tissue sections of mouse spleen and skin stained with Panx1 and Panx3 specific antibodies, respectively [11].

It is possible that the complex N-glycosylated forms of the pannexins would be localized preferentially at the cell surface, but the protruding carbohydrate moieties would act as steric hindrance to any possible docking of two pannexin hemichannels to form a gap junction-like structure. This may explain in part why Panx1 was initially reported to form gap junctions in paired frog oocytes, since glycosylation modifications in frog cells are quite different from those in mammalian cells [25]. This may also be true of any over-expression system that can generate enough unglycosylated pannexins that can traffic to the cell surface (although less efficiently than the glycosylated isoforms), and dock with an adjacent pannexin channel in a neighboring cell. Previous experiments on Panx1 expressing cells treated with tunicamycin have shown an increase in the junctional conductance [39]. However, over-expression of N-glycosylation deficient mutants of Panx1 and 3 in HeLa cells did not enable dye transfer between cells [38]. A caveat to this finding is that some populations of HeLa cells have been shown to express basal levels of Panx1 that could potentially heteromerize with the unglycosylated mutant forms of the protein and provide the glycosylation hindrance that would prevent them from docking into gap junction-like channels.

A potential dual function of pannexins forming both single membrane channels and functional gap junction channels would be reminiscent of innexins, the invertebrate gap junction proteins that in many cases are capable of that dual purpose [40]. To the best of our knowledge, innexins, similar to connexins, have not been shown to be modified by glycosylation. There are only three reports of predicted glycosylation sites on *Caenorhabditis elegans*, Innexin 7, 12 and 16 based on UniProtKB/SwissProt, but there is no evidence of actual glycosylation of the innexin proteins [41]. This lack of glycosylation would in theory allow innexins to "choose" between forming a hemichannel for paracrine function or a gap junction channel for inter-cellular communication.

There have been no reports so far of pannexin gap junction-like channels in vivo, so we will have to wait until that evidence is presented, perhaps in tissues where the unglycosylated forms of the pannexins are abundantly expressed at the cell surface. The evidence up to this point, however, favors the position that the main function of pannexins is the formation of functional single membrane channels and not gap junctions [2].

3. Caspase cleavage

The release of nucleotides, such as ATP and UTP from apoptotic cells is critical for phagocytosis [42]. Caspases play important roles, not only in the initiation of apoptosis but also in the release of nucleotides from apoptotic cells. Caspase cleavage of the carboxyl terminus of Panx1 has been reported as a means for activating channel opening. Caspase 3 and 7 cleave the human isoform of Panx1 (hPANX1) at residues 376–379, resulting in a constitutively active and open channel that releases ATP as a 'find-me" signal from apoptotic immune cells for macrophage recruitment to the site [16]. This signal is critical for the proper clearance of dead cells by monocytes and plays an important role in normal immunity. This gating mechanism, being irreversible and active during apoptosis, may be exclusive of dying immune cells. Although pannexins have been linked to cell death events in ischemia, epileptic seizures, and death of enteric neurons in colitis [13,19,21,43], it may not necessarily be through an apoptotic event, but rather a form of necrosis activated directly or indirectly by the Panx1 channel.

Furthermore, the cleaved portion of the C-terminus of hPANX1 that is released by caspase cleavage appears to be lodged in the C-terminal face of the pore, presumably plugging it in a "ball and chain" type of fashion [44]. Sandilos et al. demonstrated that by using proteolytic cleavage, and without additional apoptotic components, that deleting this critical region of the C-terminus just distal to the caspase site resulted in an activated hPANX1 channel. To prove that the C-terminal peptide was indeed the pore blocker, exogenous peptide added to the cell could block the cleaved channel at negative membrane potentials [44]. It remains to be seen if this activation procedure is also present in other Panx1-expressing cell types undergoing apoptosis, as well as in other species, since mouse Panx1 and human PANX1 show some sequence divergence in this area of the C-terminus. It will also be important to know whether the other two pannexins are also potential substrates for caspase cleavage and activation. In other systems that do not involve activated caspases present in the cell, it would be suspected that other mechanisms must be at play for relieving the putative pore block by the C-terminus. This likely occurs in a reversible and regulated manner to allow for controlled gating of pannexin channels under physiological conditions, and the best known candidates are S-nitrosylation and phosphorylation.

4. S-nitrosylation

Over the last decade or so it has become appreciated that phosphorylation is not the major game in town as a means of regulating proteins through posttranslational modifications. The addition of nitrosyl groups to specific cysteine residues (S-nitrosylation) is now considered an important posttranslational modification in both physiology and disease [45]. There are several theoretical ways in which the C-terminus could reversibly interact with the channel pore to gate pannexins. Some examples of these are transient post-translational modifications including nitrosylation and phosphorylation (see below). It is now well established that numerous proteins undergo addition of nitrosyl groups to cysteine and/or tyrosine residues as a means of regulating their function, but also that S-nitrosylation may play a prominent role in several neurodegenerative diseases [45].

Panx1 channels are activated under ischemic conditions that mimic stroke [23,43,46]. Zhang and colleagues [46] reported that blockers of nitric oxide synthase (nNOS) could prevent activation of Panx1 during exposure to O_2/glucose deprivation (OGD), assayed as dye efflux from cultured neurons [47]. They further showed that redox scavengers (DTT and GSH) were potent inhibitors of Panx1 channel opening. Together, this suggested that OGD increased NO production and activated Panx1, probably through nitrosylation of the protein. In these studies, the sites of nitrosylation on the Panx1 protein were not investigated. Several subsequent studies have looked at the impact of intracellular cysteine residues (putative sites of S-nitrosylation) on modulation of Panx1 gating.

Early studies investigating the potential role of S-nitrosylation on Panx1 opening involved site-directed mutagenesis of cysteine residues. Targeted cysteines were located in intracellular and transmembrane domains, as well as in the presumed pore-forming region. Ectopic expression of zebrafish Panx1 in neuro2A (N2A) cells with a C282W mutation (a residue in the 4th transmembrane spanning region) lead to a channel with significantly reduced function, characterized by the appearance of novel sub-conductance levels, reduced current amplitudes, and a lower rate of dye efflux [48]. Subsequent investigations analyzed the effects of mutating different cysteines throughout the Panx1 protein. Notably, the murine Panx1 C40S and C346S mutations resulted in constitutively

Table 1
Reported sites for posttranslational modifications of pannexins.

Modification	Panx1	Panx2	Panx3	References
N-glycosylation	N254	N86 (predicted)	N71	[4,11] (all three Panx) [36] (only Panx1)
Caspase cleavage	376–379			[16]
S-nitrosylation	C40, C346			[51]
Phosphorylation	Y308 (predicted)	S514		[54] (Panx1) [55] (Panx2)

open channels that induced death of oocytes upon overexpression [49]. On the other hand, C215S and C227S mutations (also transmembrane cysteines) did not alter Panx1 activation [50]. Finally, substituting the four extracellular cysteines (C66S, C84S, C245S, C264S) did not alter Panx1 function [50]. Together, these mutagenesis studies suggest that cysteines may be important for channel regulation, but a direct role for these residues in regulating channel gating/function under physiological (or pathophysiological) conditions has not been demonstrated.

The simultaneous (or sequential) nitrosylation of multiple cysteine residues may be important for regulation of Panx1 functions. For example, Lohman et al. [51], using both expression of Panx1 in HEK 293 cells or mouse aortic endothelial cells, showed that the NO donor, GSNO, potently inhibited ATP release through the channel. They further demonstrated that mutation of both C40 and C346 (to alanine) was required to prevent S-nitrosylation of the channel, where mutation of either cysteine alone could not. Interestingly, in the studies by Bunse et al. [49,50], mutation of these two cysteines (to serine) generated a constitutively open channel. The reasons for these discrepancies are not clear, but could reflect the observations that different cell types can express dramatically different proportions of Panx1 isoforms (i.e. N-glycosylated forms, splice variants). It is not known how post-translational modifications can interact to alter pannexin functions. However, in all of the cysteine mutagenesis studies to date, a physiological stimulus has not been found that alters Panx1 function through nitrosylation.

5. Phosphorylation

There are several predicted phosphorylation sites on S, T or Y residues, including putative sites for PKC, PKA and CaMKII [11,52]. Despite this, the evidence for regulation of pannexin channels by phosphorylation is currently indirect. A few studies have shown that pharmacological antagonists of kinases can alter Panx1 activation, as well as alter the amount of phospho-Panx1 that is present. However, a causal demonstration that a specific kinase phosphorylates a specific residue on Panx1 has not been shown.

Two studies have suggested that Panx1 can be opened by the activity of sarcoma (Src) family kinases (SFK) in response to ionotropic receptors. In Panx1 and P2X7 expressing J778 macrophages, the SFK inhibitor, PP2 prevented activation of Panx1 by BzATP. A targeted interfering peptide of the P2X7 receptor's proline rich C-terminal was also a potent blocker of Panx1 opening, presumably through its action on preventing SFK and P2X7 interactions [53]. The second study used a similar strategy to show that the N-methyl-D-aspartate receptor (NMDAR) in rodent hippocampal brain slices links to Panx1 through SFKs. PP2 blocked activation of Panx1 induced by anoxia, which is an NMDAR-dependent process. Furthermore, an interfering peptide that comprised amino acids 305–318 of the Panx1 C-terminal, including a presumed SFK consensus phosphorylation site of Panx1 (Y308), was also a potent blocker of anoxia-induced Panx1 opening [54]. Thus, it appears that SFKs can open Panx1 channels initiated by two different ionotropic receptors (P2X7 and NMDAR). Direct phosphorylation of Panx1 (at Y308) by SFKs has not been demonstrated and remains an open question.

To the best of our knowledge the only study to date that shows a change in phosphoserine and phosphothreonine levels of Panx1 came from the Saez group [9]. They showed an interesting series of events where ATP acting on P2Y receptors (probably P2Y1) opens Panx1 to potentiate muscle contraction. This was associated with Ca^{2+} and glucose influx through Panx1. While potentiation was accompanied by increased phospho-Panx1 protein levels, as assayed by Western blot on immunoprecipitated Panx1, the kinases involved were not identified. Furthermore, the exact S and T residues remain unidentified. It will be interesting to determine if P2Y1 receptors are exerting their activation effect on Panx1 through recruitment of SFKs or another kinase.

There is currently no evidence for phosphorylation of Panx3, though it likely occurs there. Recently, Panx2 was found to be phosphorylated at S514 in cortical neurons (Fig. 1 and Table 1) by a NDR (nuclear Dbf2-related) kinase, NDR1/2 [55]. Panx2 was identified in a kinase substrate pull-down assay, along with four other proteins. While the functional consequences of Panx2 phosphorylation at S514 is still unknown, it may play a role in regulated neurite outgrowth and dendritic spine dynamics-one of the proposed roles of substrates of NDR1/2.

6. Conclusions

An interesting consensus was reached during our roundtable discussion at the International Gap Junction Conference upon which this article is based. The group of connexin and pannexin leaders concluded that very little is actually known about pannexin post-translational modifications. To us, this is somewhat surprising because the channels were first discovered more than a decade ago. While we do know that N-glycosylation is likely important for trafficking of Panx1, and possibly for restricting intercellular junction formation, the majority of other potential post-translational modifications are poorly understood. This is perhaps best emphasized by the apparently conflicting outcome of cysteine modifications reported by several groups. Thus, the challenge of the next decade or so is to precisely define the functional outcome of pannexin post-translational modifications under physiological and pathophysiological conditions. One area of initial focus may be on the modification of the Panx1 C-terminal region, which many groups have suggested is critical in gating the channels.

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